

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**  
(Attorney Docket No. 03-769/RDID02098US)

In the Application of:	)	
	)	
Enno Adema	)	Examiner: Christine E. Foster
	)	
Serial No.: 10/652,372	)	Art Unit: 1641
	)	
Filing Date: 08/29/2003	)	Confirmation No.: 1588
	)	
For: Improvement of Specificity in the	)	
Determination of Antithrombin	)	

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

**DECLARATION OF DR. ENNO ADEMA**

1. I am the inventor of the claimed subject matter of the above-referenced patent application. My Curriculum Vitae is attached as Exhibit A.
2. I have reviewed the recent Office Action for the application (mailed March 3, 2010), as well as the prior art cited in the Office Action.
3. I understand that the Examiner considers that the claimed invention was obvious over a combination of the prior art that generally teaches the determination of thrombin in the presence and absence of heparin, *i.e.*, Plattner, *et al.*, US 4,219,497 ("Plattner") and Philo, *et al.*, Br. J. Haematol. 1982 ("Philo"). This prior art, however, teaches that the determination of thrombin in the absence of heparin is conducted on a separate reaction volume than the determination conducted in the presence of heparin.

*E*

4. The Examiner also cites a number of prior art references teaching the detection of two different analytes in the same reaction volume. *See* Furatu (EP 0041366) ("Furatu"), Morris, *et al.* (US 4,214,987) ("Morris") and Akhavan-Tafti, *et al.* (US 6,068,979) ("Akhavan-Tafti").

5. Plattner teaches a method that is different than what is presently claimed. In particular, Plattner does not teach the measurement of AT in the presence of fast acting inhibitors, such as hirudin. Instead, Plattner teaches that AT is measured in the presence of "other plasma proteins which can also inhibit thrombin." (col. 6, lns. 51-52). None of these proteins are described in Plattner, but one of skill in the art would understand that these proteins as slow inhibitors of thrombin. Such inhibitors have been described by Lebreton de Vonne and Mouray, *Int. J. Biochem* 1980; 12:479-84. (Ex. B)

6. Plattner teaches the measurement of AT-III in the presence of heparin as an "entity distinct from the 'progressive antithrombin activity' which is measured in the absence of heparin." (col. 6, lns 52-55) From these two determinations, Plattner states that "one can clearly identify a defect in the anticoagulation system as one associated with AT-III rather than other protein inhibiting mechanisms." (col. 6, lns. 55-56)

7. The measurement of AT-III activity (measurement with heparin) proceeds at a rate that would make the presence of the other proteins inconsequential in the assay. Indeed Plattner states, "[t]he presence of heparin increases the rate of reaction of AT with such proteases approximately 100-fold, *making AT the only plasma component involved in this rapid reaction*" (emphasis added).

8. The present invention is distinctly different from the method described in Plattner because the first reaction *measures the presence of the inhibitor, not AT, as the only plasma*

*component involved* in the reaction. As recited in step (a) of claim 1, the conditions in the reaction mixture are such that thrombin essentially does not interact with AT, but interacts with the inhibitor.

9. Hirudin, and other drugs that inhibit thrombin, act differently in an AT assay than the slow reacting plasma proteins. Hirudin inhibits thrombin at the same fast rate regardless of the presence of heparin. Therefore, when hirudin is present in a sample, AT *is not the only component in plasma* that is involved in the inhibition of thrombin, even in the presence of heparin. Moreover, Plattner does not teach anything about measuring thrombin in the presence of an inhibitor for thrombin under conditions that thrombin does not interact with AT. In Plattner, it is expected that the thrombin interacts with AT in the presence of heparin, and also in the absence of heparin in a “progressive antithrombin assay” that is mentioned in Plattner.

10. Although it may not be explicitly stated, one can infer that Plattner also teaches measurement of these “other protein inhibiting mechanisms” as follows: two measurements of thrombin are made:

- a. with heparin – this reaction proceeds very quickly and all of the AT in the sample can be expected to interact with thrombin. Excess thrombin is measured, and “AT is the only plasma protein involved in this rapid reaction.” (col. 6, lns. 36-37).
- b. progressive antithrombin activity – in order to determine the inhibition by other plasma proteins, the reaction must proceed longer than it takes for thrombin-AT interaction in the presence of heparin. Excess thrombin is measured, which can be compared to the measurement of excess thrombin in the presence of heparin.

The antithrombin activity determined under “b” minus the antithrombin activity under “a” equals the antithrombin activity of other protein inhibiting mechanisms. An inhibitor like hirudin would not be recognized as “antithrombin activity of other protein inhibiting mechanisms”, rather it would be recognized as AT III. Since hirudin is not AT III, the result obtained by following Plattner is false high.

11. The Examiner cites Philo for essentially the same proposition as Plattner. Philo teaches assays with and without heparin. In the progressive antithrombin assay of Philo, the reaction proceeds to completion after an incubation time of one hour. As in Plattner fast acting thrombin inhibitors will affect the result with and without heparin. There is no possibility to distinguish between AT and a fast acting inhibitor and, thus, no possibility to determine the true AT level in the presence of such inhibitors.

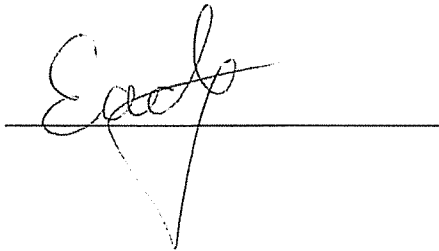
12. With regard to the Examiner’s reference to Morris, Furata, and Akhavan-Tafti, while these references teach two analysis of the same sample, none of the references teach the determination of a single analyte, using the same reagents, in the presence of an inhibitor. For instance, Furata, teaches the determination of GOT first, then the level of GPT, to obtain the level of GPT. This detection of two different analytes is different than the presently claimed method where only a single analyte (AT) is measured and the interfering substance is not determined.

13. Morris describe a decision tree where, depending on the first result (the antinuclear antibody test), other tests are performed. The tests are performed sequentially and are unrelated, other than the sense of a clinical/diagnostic logic. Akhavan-Tafti concerns the visualization of different analytes on a solid phase through the use of different probes. The claimed assay is homogeneous and uses a single probe (thrombin in combination with its chromogenic substrate).

14. The claimed invention is an alternative to assays that use Factor Xa as the binding partner for ATIII. While Factor Xa is not inhibited by hirudin and similar drugs that directly inhibit thrombin, many new drugs are being developed, some of which are inhibitors of Factor Xa (*e.g.*, Fondaparinux; *see* Ex. C., Braun, *et al.*, and K. Hickey *et al.*, Figure 3, GTH Congress 2008). These substances lead to false high AT result in Xa-based AT assays. Thus, the Xa-based AT assays suffer from the same problem. The present method is able to detect ATIII regardless of the inhibitor that may be present in the sample. Because the laboratory may not be aware which drug that a patient may have been given prior to a blood sample is taken, the use of the claimed invention is advantageous because it would allow for the determination of AT regardless of the drug.

15. I hereby declare further that all statements made herein by me to my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed:

A handwritten signature in black ink, appearing to be 'E. C. Braun', written over a horizontal line.

Date:

6-july-2010

# EXHIBIT A

ENNO ADEMA

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## PROFESSIONAL EXPERIENCE

ROCHE DIAGNOSTICS / BOEHRINGER MANNHEIM

- |   |                            |
|---|----------------------------|
| o Director Quality Assurance R&D  | Since End 2007:            |
| o Director Product Management Coagulation.  | July 2000 – End 2007       |
| o Project and project-team leader R&D   | September 1990 – June 2000 |
| o Detached for 6 months as “coagulation expert” to one of the sales teams in Bavaria. | 1996                       |

From 1990 up to 2007 the focus was on the DIAGNOSTICA STAGO products distributed by Boehringer Mannheim / Roche Diagnostics. In addition, responsible for the introduction of Tina-quant D-Dimer, Hitachi/Integra Antithrombin III, HEXAMATE Factor XIII and Tina-Quant Soluble Fibrin. I was the project leader for RCS (Roche Coagulation Systems).

## CONSULTANT FOR:

- Valio (Helsinki) June – August 1987 and April 2000.
- Bioinformation Associates (Boston) December 1985.

## EDUCATION

## MASSACHUSETTS INSTITUTE OF TECHNOLOGY

Ph.D. in Biochemical Engineering, September 1989. Thesis project on ammonium toxicity in mammalian cell culture. Member Honor Society Sigma Xi

## RUTGERS UNIVERSITY

M.S. in Chemical/Biochemical Engineering, June 1983. Thesis project on computer modeling of a tubular enzyme reactor.

## CHRISTELIJKE HOGERE TECHNISCHE SCHOOL

Ing. In Chemical Engineering, June 1981. Thesis project on characterization of air purification by compost filtration.

## **PUBLICATIONS**

Assorted scientific publications, in recent times always as “ghost writer”.

### Patents

Method for determination of coagulation parameters (DE 0665435, US 5,552,296) - abandoned

System to minimize evaporation and/or gas exchange (DE 10038350 C2) – abandoned

Improvement of specificity in the determination of anti-thrombin (DE 10239821.6) - active

## **LANGUAGES**

Fluent in Dutch, English, German with basic knowledge of French.

## **BACKGROUND**

Born 12 September 1960 in the Netherlands. Married, 1 daughter (14 years). Soaring pilot (approx 1000 hrs).



# **EXHIBIT B**

## THE ROLE OF ANTITHROMBIN III, $\alpha$ 2 MACROGLOBULIN AND $\alpha$ 1 ANTITRYPSIN IN PROGRESSIVE ANTITHROMBIN ACTIVITY OF HUMAN PLASMA

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(Received 18 December 1979)

**Abstract**—1. The results show that antithrombin III is responsible for 70% of the total antithrombin activity,  $\alpha$  2 macroglobulin 50%, and  $\alpha$  1 antitrypsin 40%.

2. There is competition among these various antithrombins.

3. As the progressive antithrombin activity is not completely cancelled by the removal of the three well known principal antithrombins, there must be in defibrinated human plasma other antithrombins responsible for about 30% of this activity. The next vital step is to identify them.

### INTRODUCTION

In human plasma thrombin is inactivated by various natural inhibitors. As fibrinogen is an important inhibitor care is taken to eliminate it before any progressive antithrombin study is undertaken. Once defibrinated, the plasma still contains several antiproteases that have progressive antithrombin activity. It is generally accepted that three plasma proteins are responsible for the majority of this activity. These are: antithrombin III (AT III) known as its name indicates, for its capacity as a thrombin inhibitor, as well as its progressive antithrombin activity, and its capacity as a cofactor of heparin (Abildgaard, 1967; Fagerhol & Abildgaard, 1970);  $\alpha$  2 macroglobulin ( $\alpha$ 2M), whose antithrombin activity is well known, either isolated or in serum (Lanchantin *et al.*, 1966; Steinbuch *et al.*, 1967, 1968); and  $\alpha$  1 antitrypsin ( $\alpha$ 1AT) (Rimon *et al.*, 1966; Matheson *et al.*, 1976; Machovich *et al.*, 1977). This study systematically investigates the progressive antithrombin activity of each of the three natural thrombin inhibitors on defibrinated human plasma. To achieve this, the progressive antithrombin activity of such a plasma was compared to that of the same plasma deprived of one of the three thrombin inhibitors: AT III,  $\alpha$ 2M, or  $\alpha$ 1AT. These activities were also compared to those of the same plasma deprived simultaneously of all three antithrombins. This way, the respective participation of each of the three proteins in the progressive antithrombin activity of plasma could be deduced within the physiological milieu of the plasma.

### MATERIALS AND METHODS

Blood from eight healthy donors at the Tours Blood Transfusion Center was collected in plastic tubes containing 1 part 3.8% sodium citrate solution to 9 parts blood. The donors were fasting. The cells were removed by centrifugation and the fibrinogen was precipitated by heating to 56°C (3 min for 3 ml) followed by centrifugation. The supernatant (defibrinated plasma) obtained was then

divided into several tubes in order to have one or all of the antiproteases under study removed by addition of specific immunoglobulins.

#### *Separation of antibodies (IgG) from rabbit specific immunosera*

Immunosera anti-AT III, anti- $\alpha$ 1AT and anti  $\alpha$ 2M were obtained from Behring Laboratories. They were precipitated by  $(\text{NH}_4)_2\text{SO}_4$  (40% final concentration). The various specific immunoglobulins thus obtained were then solubilized in NaCl 9%<sub>m</sub>, dialyzed in NaCl 9%<sub>m</sub> and concentrated by a factor of 6.

The removal of each of the three proteins from the plasma was accomplished in the following manner: 50  $\mu$ l of anti-AT III, or anti- $\alpha$ 1AT, or anti- $\alpha$ 2M antibodies or 50  $\mu$ l of each of the three immunoglobulin solutions was added to 50  $\mu$ l of defibrinated plasma. A control of 50  $\mu$ l plasma and 50  $\mu$ l 9% NaCl solution was used. The five different tubes were sealed and placed in a 37°C water bath and agitated for 2.5 hr. The "antigen-antibody" precipitates were then eliminated by centrifugation at 15,000 rpm and the supernatants were collected. 50  $\mu$ l plasma was removed from each tube and diluted to 1/16 in a 9% NaCl solution. For each sample, the extraction of the antiprotease by its specific antibodies was verified by electroimmunodiffusion using Laurell's technique (Laurell, 1966).

It was further verified that the different antibodies used have, at the concentration used in the experiment, no significant progressive antithrombin activity.

The progressive antithrombin activity of each sample was measured on human plasma adsorbed on  $\text{BaSO}_4$  using Astrup & Darling's (1943) technique but we chose to utilize a solution of 13.3 NIH units of Stago\* thrombin per milliliter. The results are expressed in residual thrombin activity half-life as has been previously described (Lebreton de Vonne *et al.*, 1978a). Lastly, each donor's plasma was titrated for the three antiproteases by radial immunodiffusion as described by Mancini *et al.* (1965) on Behring Laboratories immunodiffusion plates.

### RESULTS

Immunological titrating of the three plasma proteins studied allows verification that the levels of each donor are normal; they are for  $\alpha$ 1AT 1.6–2.65 g/l, for  $\alpha$ 2M 1.82–2.7 g/l and for AT III 0.19–0.39 g/l. The co-

\* STAGO Laboratories, 92-Asnieres, France

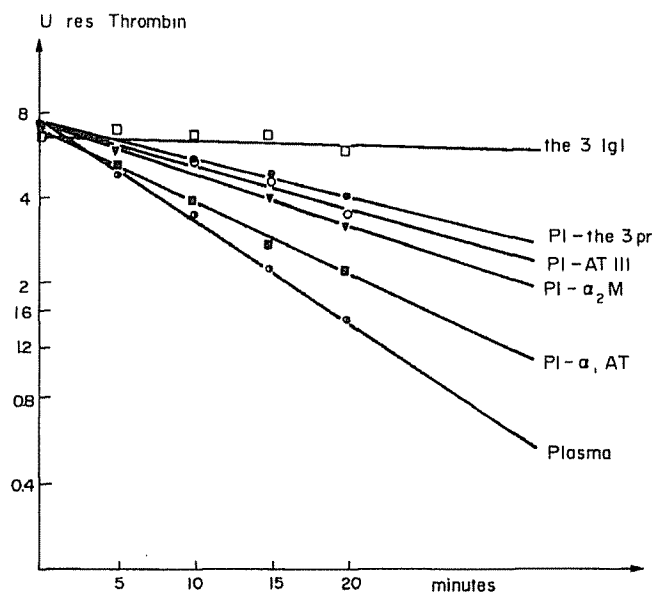


Fig. 1. Residual thrombin activity expressed in NIH Units of thrombin as a function of incubation time at 37°C. Results obtained for donor Dr's plasma and the same plasma deprived of AT III, deprived of  $\alpha$ 1AT, deprived of  $\alpha$ 2M and deprived of all 3 proteins. Results for the 3 specific immunoglobulins (IgI).

agulation time of the thrombin-plasma mixture on the adsorbed plasma becomes longer, demonstrating antithrombin progressive inhibition for the control plasma as well as for the different plasmas deprived of one or more proteins. Fig. 1 shows the results obtained from one donor's plasma. The longest increases in time which were very close were for the plasma missing all three anti-thrombins, and the plasma without AT III, showing a particularly intense progressive antithrombin activity. This semi-logarithmic graph of the progressive anti-thrombin activity indicates the thrombin activity half-lives of the different plasmas taken from the same subject. Table 1 gives the half-lives for each plasma from the same donor and corresponds to the curves shown in Fig. 1.

The verification of the antiprotease elimination performed on the plasmas from this same donor are represented in Fig. 2.

The results obtained for the eight donors tested are expressed in Table 2 as the percentage of the mean residual thrombin activity value for each of the plasmas without one or three antiproteases and as the

inhibition potentiality of thrombin activity for each extracted antiprotease.

#### DISCUSSION

The small number of subjects studied (8 cases) is insufficient to establish a valid correlation between the residual thrombin activity half-life and the level of each of the three antiproteases. The values found, however, are within the range of those given by Abildgaard *et al.* (1970) and Gomperts *et al.* (1977) as regards the proportionality existing between the concentration of AT III and the progressive antithrombin activity and the total absence of correlation between the  $\alpha$ 2M concentration or the  $\alpha$ 1AT concentration and the same activity.

The authors have previously shown that, in normal subjects, the progressive antithrombin activity is the result of simultaneous activity by the three antithrombins studied excepting fibrinogen; while in the nephrotic subject, this activity is proportional to the amount of  $\alpha$ 2M, the two other proteins having disap-

Table 1. Half-lives of the residual thrombin activity obtained for donor Dr's plasma, by representation of the decrease in progressive antithrombin activity which is exponential expressed in semi-logarithmic coordinates.

	Half-lives of the Residual thrombin Activity	Difference by reference to the control plasma
Dr's Plasma	8'35"	
Plasma—AT III	21'20"	+ 12'45"
Plasma— $\alpha$ 1AT	12'35"	+ 4'
Plasma— $\alpha$ 2M	18'20"	+ 9'45"
Plasma—the 3 Proteins	25'50"	+ 17'15"

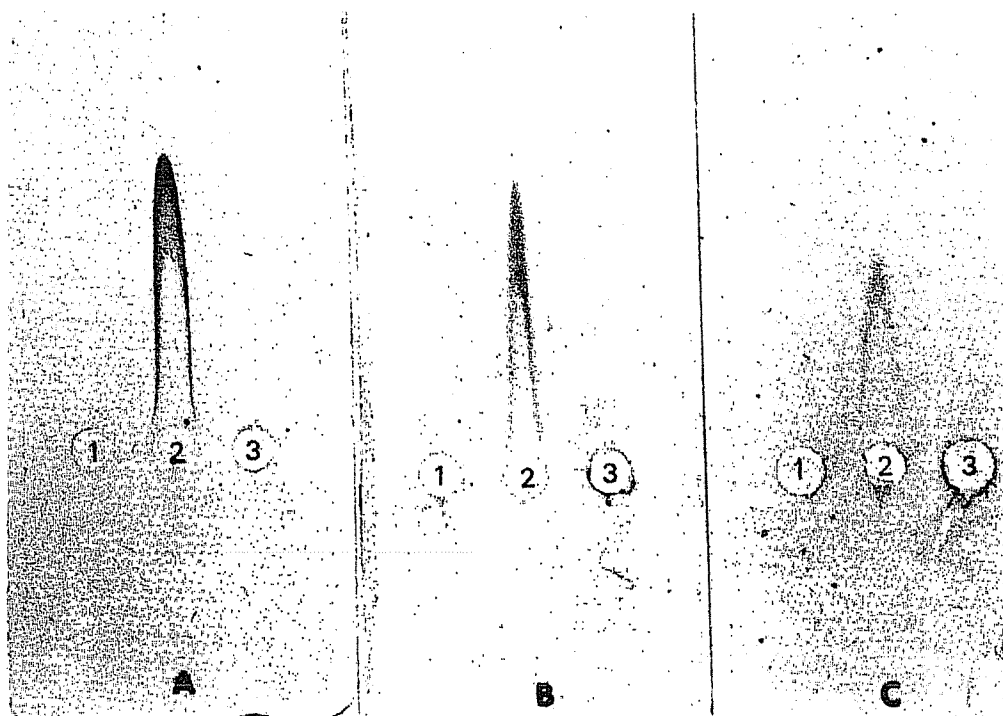


Fig. 2. Electroimmunodiffusion using Laurell's technique: A—Agarose containing anti- $\alpha$ 1AT immunserum; B—Agarose containing anti- $\alpha$ 2M immunserum; C—Agarose containing anti-AT III immunserum. Well 2—plasma from donor Dr; Well 1—Dr's plasma with corresponding protein extracted; Well 3—Dr's plasma with all three proteins extracted.

Table 2. Mean values and limits for eight donors from the Tours Blood Transfusion Center whose rates of extraction for the different antiproteases was total or above 92%.

	Half-lives of the control plasma	Difference by reference to the control plasma	Residual thrombin activity of plasma	Inhibition potentiality of thrombin activity for each extracted antiprotease
Plasma	7'50" (6'-9'20")			
Plasma—AT III	24'38"	+16'48" (12'05"—24'40")	67.8%	32.2%
Plasma— $\alpha$ 1AT	12'34"	+4'44" (1'55"—11'10")	61%	39%
Plasma— $\alpha$ 2M	16'10"	+8'20" (4'-10'55")	48%	52%
Plasma—the 3 proteins	22'50"	+15' (5'05"—18'10")	33.9%	66.1%

peared in part from general circulation (Lebreton de Vonne *et al.*, 1978b). It is possible to argue that plasma defibrination by heat partially destroys certain proteins and plasma thus treated is not exactly physiological. On the one hand, other defibrination methods, addition of small quantities of thrombin (Seegers *et al.*, 1952) or adsorption on bentonite (Soulier, 1959), could not have been used in the study as several adsorptions would have been necessary and that would have inevitably provoked the loss of other coagulation factors. On the other hand, it has been verified in our laboratory that treatment at 56°C of the three proteins studied affects neither their antigenicity nor their concentration; but it is not possible, in the present conditions, to affirm there is unchanged antithrombin capacity.

The role of each protein studied with respect to the progressive antithrombin activity is quite different (Tables 1 and 2). AT III has an undeniably strong antithrombin activity in the plasma as evidenced by its elimination provoking a noticeable decrease (67.8%) in the antithrombin capacity of the plasma. The results here are, quite similar to those of Fagerhol *et al.* (1970) on plasma and Learned *et al.* (1976) on the isolated molecule. They, using a different technique, showed AT III was responsible for 70–75% of the progressive antithrombin activity in the man, whereas Lane *et al.* (1975) attributed 50% of this activity to AT III, the two other antiproteases sharing the rest of the activity equally. The progressive antithrombin activity of  $\alpha$ 2M represents more than 50% of the total defibrinated plasma antithrombin activity: these results corroborate those found by Steinbuch *et al.* (1968), who evaluated the  $\alpha$ 2M participation between 30 and 60% of the physiological antithrombin activity of normal human serum. This important antithrombin capability of  $\alpha$ 2M explained its major role in nephrotic patients who have lost the other antithrombins (AT III and  $\alpha$ 1AT) into the urinary system (Lebreton de Vonne & Mouray, 1978).

Lastly,  $\alpha$ 1AT was responsible for roughly 40% of the progressive antithrombin activity of defibrinated plasma; however, individual variations intervened more often than for the two other antiproteases. This was most likely in relation to the amount in plasma of  $\alpha$ 1AT and perhaps different phenotypes. In effect, the

extremes vary from 2 min to more than 11 min, while for the two other proteins the maximum value barely exceeded double their minimum. In addition, it should be noted that elimination of AT III by itself, or the three proteins simultaneously, resulted in a diminution almost identical to 2/3 of the total antithrombin activity. The influence of  $\alpha$ 1AT in this phenomena is without a doubt non negligible.

It should be noted that the respective activities of the three antithrombins was not additive. There exists therefore a competition among the three antithrombins present. It is seen that using up AT III did not permit  $\alpha$ 1AT and  $\alpha$ 2M to fully express their inhibition capability, since the sum of their potential represents 90% and there was only found 32% inhibition, or 1/3 of the global potential of the two antiproteases. Elimination of  $\alpha$ 1AT or  $\alpha$ 2M respectively only allowed half of the potential of  $\alpha$ 2M and AT III or  $\alpha$ 1AT and AT III. This confirmed the importance of competition among these antiproteases. Moreover, it was evident that the efficiency of these three antithrombins is not only a function of their concentration, but also of their affinity with respect to thrombin (Abildgaard *et al.*, 1970). It therefore seems more logical to speak of inhibition potential than inhibitor capability. All these results contradict a recent *in vitro* study done by Downing *et al.* (1978) on the three isolated antiproteases; he calculated that for  $\alpha$ 1AT, an antithrombin activity triple that of  $\alpha$ 2M and five times less than that of AT III, taking into consideration the molar plasma concentrations of the three antiproteases. These authors did not, however, consider the competition that exists in nature among the different inhibitors.

Finally, removing the three principally known antithrombins does not annul the progressive antithrombin activity. We are therefore forced to admit that human plasma contains other antiproteases capable of assuring one third of the total antithrombin activity. Miller-Andersson *et al.* (1973) have shown that  $\beta$ -lipoproteins have antithrombin capability. The problem now is to study in what measure these lipoproteins participate in progressive antithrombin activity, in normal plasma and in pathological cases; and if there exists, in addition, other antithrombins as yet unidentified.

**Acknowledgements**—The authors wish to thank Miss Bellver and the Blood Transfusion Center (Professor Arnaud) for the generous gift of materials and Dr Vairel for his encouragement and his valuable discussions.

This work was supported by a grant from INSERM (C.R.L. No. 76.1.070.3).

#### REFERENCES

- ABILDGAARD U. (1967) Inhibition of the thrombin-fibrinogen reaction by antithrombin III, studied by N-terminal analysis. *Scand. J. clin. lab. Invest.* **20**, 205–216.
- ABILDGAARD U., FAGERHOL M. K. & EGEGERG O. (1970) Comparison of progressive antithrombin activity and the concentrations of three thrombin inhibitors in human plasma. *Scand. J. clin. Invest.* **26**, 349–354.
- ASTRUP T. & DARLING S. (1943) Measurement and properties of antithrombin. *Acta Physiol. scand.* **4**, 293–394.
- DOWNING M. R., BLOOM J. W. & MANN K. G. (1978) Comparison of the inhibition of thrombin by three plasma protease inhibitors. *Biochemistry* **17**, 2649–2653.
- FAGERHOL M. D. & ABILDGAARD U. (1970) Immunological studies on human antithrombin III. Influence of age, sex and use of oral contraceptives on serum concentration. *Scand. J. Haemat.* **7**, 10–17.
- GOMPERS E. D., ZUCKER M., FEESEY M., RUSSEL D., SALANT D., JOFFE B. I., MENDELSON D. & SEFTEL H. (1977) Antithrombin functional activity after a fatty meal in normal subjects, familial hyperlipidemia and nephrotic syndrome. *J. Lab. clin. Med.* **90**, 529–535.
- LANCHANTIN G. F., PLESSET M. L., FRIEDMAN I. A. & HART D. W. (1966) Dissociation of esterolytic and clotting activation of thrombin by trypsin-binding macroglobulin. *Proc. Soc. exp. Biol., New York* **121**, 444–449.
- LANE J. L., BIRD P. & RIZZA C. R. (1975) A new assay for the measurement of total progressive antithrombin. *Br. J. Haemat.* **30**, 103–115.
- LAURELL C. B. (1966) Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. *Ann. Biochem.* **15**, 45–52.
- LEARNED L. A., BLOOM J. W. & HUNTER M. J. (1976) The antithrombin activity of  $\alpha 1$  protease inhibitor: the antitrypsin activity of antithrombin III. *Thromb. Res.* **8**, 99–109.
- LEBRETON DE VONNE T., VERSAVEL C. & MOURAY H. (1978a) Etude de l'activité antithrombinique progressive du plasma de lapin. Rôle des  $\alpha$  macroglobulines. *C.r. Acad. Sci. Paris* **286**, 1395–1397.
- LEBRETON DE VONNE T. & MOURAY H. (1978b) Human  $\alpha 2$  macroglobulin and its antitryptic and antithrombin activities in serum and plasma. *Clinica chim. Acta* **90**, 83–85.
- MACHOVICH R., BORSODI A., BLASKO G. & ORAKZAI S. A. (1977) Inactivation of  $\alpha$  and  $\beta$ -thrombin by antithrombin III,  $\alpha 2$  macroglobulin and  $\alpha 1$ -Proteinase inhibitor. *Biochem. J.* **167**, 393–398.
- MANCINI G., CARBONARA A. O. & HEREMANS J. F. (1965) Immunochemical quantitation of antigen by single radial diffusion. *Immunochemistry* **2**, 235–254.
- MATHESON N. R. & TRAVIS J. (1976) Inactivation of human thrombin in the presence of  $\alpha 1$  proteinase inhibitor. *Biochem. J.* **159**, 495–502.
- MILLER-ANDERSSON M., ANDERSSON L. O. & BORG H. (1973) IVth International Congress on Thrombosis and Haemostasis (June 19 and 20, 1973, Vienna, Austria) abstract 181, p. 216.
- RIMON A., SHAMASH Y. & SHAPIRO B. (1966) The plasmin inhibitor of human plasma; IV its action on plasmin, chymotrypsin and thrombin. *J. biol. Chem.* **241**, 5102–5108.
- STEEGERS W. H., MILLER K. D., ANDREWS E. B. & MURPHY R. C. (1952) Fundamental interactions and the effect of storage, ether, absorbants and blood clotting on plasma antithrombin activity. *Am. J. Physiol.* **169**, 700–711.
- SOUJIER J. P. (1959) Un nouvel adsorbant des facteurs de coagulation: la bentonite. *Rev. Hemat.* **14**, 26–40.
- STEINBUCH M., BLATRIX C. & JOSSE F. (1967)  $\alpha 2$  macroglobulin as progressive antithrombin. *Nature* **216**, 500–501.
- STEINBUCH M., BLATRIX C. & JOSSE F. (1968) Action anti-protéase de l' $\alpha 2$  macroglobuline II—son rôle d'antithrombine progressive. *Rev. Franç. Et. Clin. Biol.* **13**, 179–186.

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# EXHIBIT C

# Clinical evaluation of a new FXa-based Antithrombin assay on Sysmex® CA-1500 System

Kieron Hickey<sup>a</sup>, Peter Cooper<sup>a</sup>, Steve Kitchen<sup>a</sup>, Mechthild Merz<sup>b</sup> and Martina Böhm-Weigert<sup>b</sup>

<sup>a</sup> Sheffield Haemophilia and Thrombosis Centre, Royal Hallamshire Hospital, Sheffield, United Kingdom

<sup>b</sup> Dade Behring Marburg GmbH – A Siemens Company, Marburg, Germany

## Objectives:

Screening for Antithrombin (AT) deficiency is done with FIIa- and FXa-based methods. FXa-based methods are often believed to be superior to FIIa-based methods because of the absence of Heparin Cofactor II interference. On the other hand, FIIa-based assays have demonstrated in some patients higher sensitivity for certain AT type II defects. In this study a new FXa-based AT assay (*Innovance*® Antithrombin – Product under development, not available for sale) from Dade Behring Marburg GmbH – A Siemens Company, Marburg, Germany was evaluated and compared to the established FIIa-based Berichrom® Antithrombin III (A) assay from the same company.

## Results and Discussion:

### 1. Precision of *Innovance*® Antithrombin

As depicted in Figure 1, the precision of the new assay is excellent, showing for controls and plasma pools within-device CVs (total CVs), repeatability CVs (within-run CVs) and between-run CVs between 3.0-6.4%, 1.1-4.2, 0.9% and 0.4-2.4%, respectively.

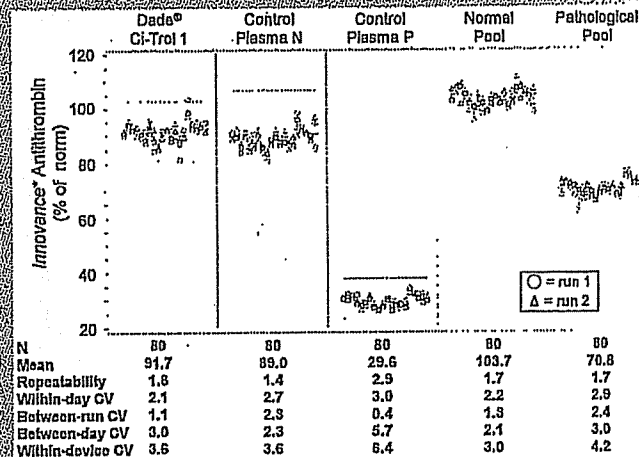


Figure 1: Precision data for *Innovance*® Antithrombin

### 2. Method Comparison between *Innovance*® Antithrombin and Berichrom® Antithrombin III (A)

The *Innovance*® Antithrombin assay was well correlated to the Berichrom® Antithrombin III (A) assay as demonstrated by a Pearson correlation coefficient of 0.96, a slope of 0.98 and an intercept of 1.56% ( $n=120$ , Figure 2). For the correlation analysis, samples with FIIa and FXa inhibitors were excluded (see Results and Discussion, point 3).

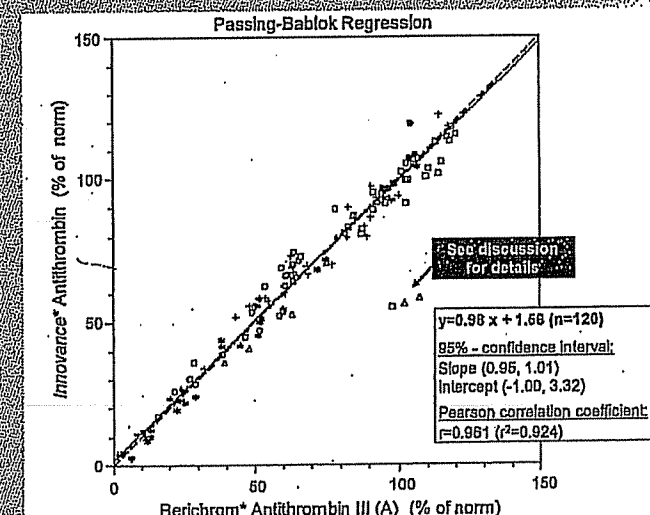


Figure 2: Comparison between *Innovance*® Antithrombin

## Design and Methods:

Samples were measured from patients with hereditary AT deficiency ( $n=7$ ), patients with acquired AT deficiency ( $n=22$ ), patients with normal AT activity ( $n=30$ ) and patients receiving heparin ( $n=25$ ) or Lepirudin ( $n=6$ ). In addition, diluted plasma samples ( $n=30$ ), plasma spiked with AT concentrate ( $n=6$ , target concentration: 80 – 120 %) and plasma spiked with Fondaparinux ( $n=12$ , target concentration: 1.2-1.8 µg/mL) were tested. The precision of the new assay was evaluated by testing three commercial controls and two plasma pools on 20 days (two runs per day, two measurements per run). All measurements were performed on Sysmex® CA-1500 System.

All patients with hereditary AT deficiency were correctly identified with *Innovance*® Antithrombin whereas 2 out of 7 patients had normal AT activity with Berichrom® Antithrombin III (A) (Figure 2). Both of these patients carry the same Pro73Leu mutation, which impairs interaction of AT with heparin. Interestingly, the two patients were identified as pathological if Berichrom® Antithrombin III (A) was performed with decreased incubation time of the diluted samples with thrombin. The increase of sensitivity for the detection of type II HBS (heparin binding site) defects by decreasing the incubation time has been demonstrated earlier<sup>2,4</sup>.

One patient demonstrated normal AT activity with Berichrom® Antithrombin III (A) but deficient AT activity using *Innovance*® Antithrombin (97.9 and 55.2 % respectively). This sample was pathological (71 %) using the Berichrom® Antithrombin III (A) assay with decreased incubation time. Two dimensional electrophoresis with heparin showed a normal fast moving peak and an abnormal slow moving peak, indicating heterozygous type II HBS deficiency.

### 3. Influence of Factor Xa and Factor IIa inhibitors on *Innovance*® Antithrombin and Berichrom® Antithrombin III (A) assays

As depicted in Figure 3, samples from patients under therapy with the thrombin inhibitor Lepirudin demonstrated slightly higher values in the FIIa-based Berichrom® Antithrombin III (A) assay compared to the FXa-based *Innovance*® Antithrombin assay. Fondaparinux (FXa inhibitor) spiked samples showed 10-50 % higher values using *Innovance*® Antithrombin assay compared to the Berichrom® Antithrombin III (A) assay.

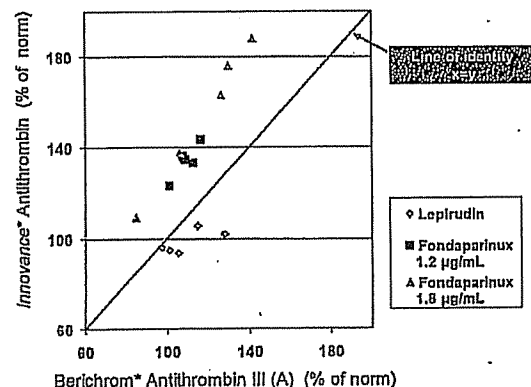


Figure 3: Influence of Lepirudin and Fondaparinux on AT assays

## Conclusions:

*Innovance*® Antithrombin demonstrated high precision and excellent comparability to the Berichrom® Antithrombin III (A) method. *Innovance*® Antithrombin had higher sensitivity to a type IIb AT deficiency (Pro73Leu mutation affecting heparin-binding site) than the FIIa-based method. The *Innovance*® Antithrombin assay is easy to perform in particular because of the ready-to-use reagents.

## References:

- Bohner J, von Pape KW, Blaurock M. Thrombin-based antithrombin assays show overestimation of antithrombin III activity in patients on heparin therapy due to heparin cofactor II influence. *Thromb Haemost* 1994; 71: 280-3
- Kristiansen SR, Rasmussen B, Pedersen S, Bathum L. Detecting antithrombin deficiency may





# Clinical assessment of a new FXa-based method for Antithrombin determination

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## Objectives:

Antithrombin (AT) is a major physiological inhibitor of hemostasis, and correct assessment of AT activity is crucial to detect congenital and acquired deficiencies. The activity of AT is usually measured by chromogenic tests measuring the inhibition of FIIa or FXa in the presence of heparin. Several reports about a discrepancy between FIIa- and FXa-based methods have been published<sup>1,2</sup>. In this study, a new AT assay (*Innovance*\* Antithrombin – Product under development, not available for sale), which is based on FXa inhibition is evaluated and compared to the well-known FIIa-based Berichrom\* Antithrombin III (A) assay. Both assays are from Dade Behring Marburg GmbH – A Siemens Company, Marburg, Germany.

## Design and Methods:

The following patient groups were analyzed:

- patients with hereditary AT deficiency (n=4)
- patients with acquired AT deficiency (n=25)
- patients with normal AT activity (n=41)
- patients under heparin (HMWH) (n=33), Argatroban (n=5) and Fondaparinux (n=5) therapy

Furthermore, diluted plasma samples (n=30) and plasma spiked with Antithrombin concentrate (n=15) were tested. To evaluate the effect of freezing, most of the samples were tested fresh and after 1-2 weeks storage at -70 °C. The precision was determined by testing 3 commercial controls and two plasma pools. All measurements were performed on the BCS® System.

## Results and Discussion:

- In the investigated cohort, the overall correlation between *Innovance*\* Antithrombin and Berichrom\* Antithrombin III (A) was remarkably high with a Pearson correlation coefficient of 0.99, a slope of 1.01 and an intercept of 0.182 % (Figure 1, samples from patients under Argatroban and Fondaparinux therapy were excluded).
- As expected, Argatroban caused an increase in AT values in the FIIa-based Berichrom\* Antithrombin III (A) assay: (mean [range]: *Innovance*\* Antithrombin 108 % of norm [105-116 % of norm]; Berichrom\* Antithrombin III (A) > 124 % of norm) The interference of direct thrombin inhibitors in FIIa-based assays have also been reported by others<sup>3</sup>.
- Fondaparinux caused a slight increase of results in the FXa-based *Innovance*\* Antithrombin assay: (mean [range]: Berichrom\* Antithrombin III (A) 106 % of norm [96-120 % of norm]; *Innovance*\* Antithrombin 123 % of norm [108-137 % of norm])
- The comparison between fresh and frozen samples showed, that both sample types produce concordant results with Pearson correlation coefficient of 0.99 (Figure 2).
- The precision of *Innovance*\* Antithrombin was excellent with within-device CV (total CV), repeatability (within-run CV) and between-run CV of <5 % for controls and plasma pools (Figure 3).

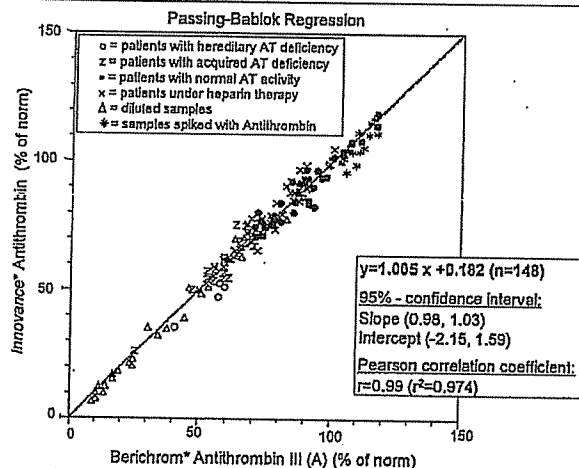


Figure 1: Comparison between *Innovance*\* Antithrombin and Berichrom\* Antithrombin III (A)

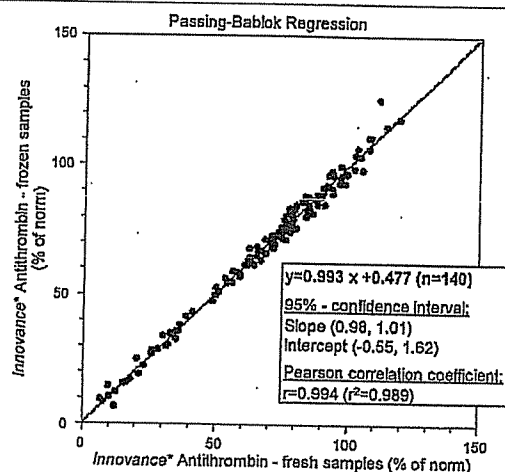


Figure 2: Comparison between fresh and frozen samples

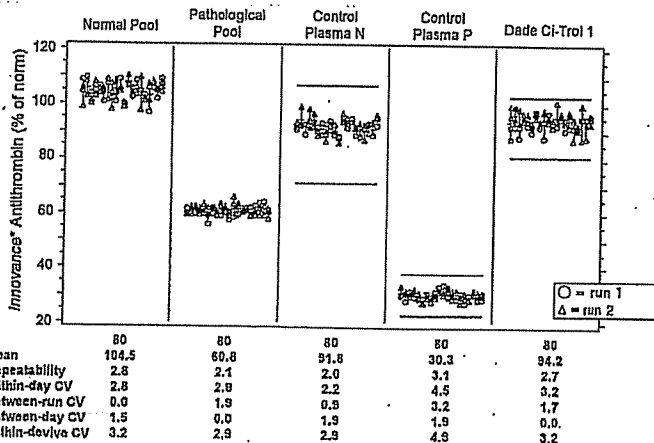


Figure 3: Precision of *Innovance*\* Antithrombin

## Conclusions:

*Innovance*\* Antithrombin is a reliable, accurate and precise AT assay. The striking advantages of this assay are the absence of interference by direct thrombin inhibitors and the ready-to-use reagents.

## References:

- <sup>1</sup> Kristensen SR, Rasnussen B, Pedersen S, Bathum L. Detecting antithrombin deficiency may be a difficult task - more than one test is necessary. *J Thromb Haemost* 2007; 5: 617-8
- <sup>2</sup> Böhner J, von Pape KW, Blaurock M. Thrombin-based antithrombin assays show overestimation of antithrombin III activity in patients on heparin therapy due to heparin cofactor II influence. *Thromb Haemost* 1994; 71: 280-3
- <sup>3</sup> Beek H, Nagel D, Pindur G, Scharrer I, Preiss A, Sailer D, Hellstern P. Measurement of antithrombin activity by thrombin-based and by factor Xa-based chromogenic substrate assays. *Blood Coagul Fibrinolysis* 2000; 11: 127-35